Apparent Inhibition by Arginine of Macrocyclic *b* Ion Formation from Singly Charged Protonated Peptides

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There is now strong evidence for the existence of macrocyclic isomers of b_n^+ ions, the formation and subsequent opening of which can lead to loss of sequence information from protonated peptides in multiple-stage tandem mass spectrometry experiments. In this study, the fragmentation patterns of protonated YARFLG and permuted isomers of the model peptide were investigated by collision-induced dissociation. Of interest was the potential influence of the arginine residue, and its position in the peptide sequence, on formation of the presumed macrocyclic b_5 ion isomer and potential loss of sequence information. We find that regardless of the sequence position (either internal or at the N- or C-terminus), only direct sequence ions or ions directly related to fragmentation of the arginine side chain are observed. (J Am Soc Mass Spectrom 2010, 21, 1322–1328) © 2010 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Collision-induced dissociation (CID) and tandem mass spectrometry are now widely used for the identification and characterization of peptides and proteins in proteomics. During low-energy CID, fragmentation of protonated peptides typically involves charge mediated unimolecular reactions, which ultimately result in cleavage of amide bonds and generation of a series of *b*, *y*, and *a* ions [1, 2]. Central to the fragmentation of protonated peptides is the mobilization and migration of protons, presumably from the most basic groups on a gas-phase peptide, to the site of an intramolecular nucleophilic attack that leads to cleavage of the amide bond. Consideration of proton mobilization and migration within this context led to the development of the mobile proton model [3, 4] of peptide fragmentation and related amide bond cleavage pathways [5–10]. A recently developed "pathways in competition" model [10] builds upon the seminal discussion of proton migration within the mobile proton model and includes consideration of the structures and reactivity of key reactive configurations and primary (post-cleavage) fragments as well as transition states and their energies.

Many experimental and theoretical studies of sequence ion formation and structure have produced evidence that N-terminal b_n type fragment ions have C-terminal oxazolone rings [5, 11] and retain much of the primary sequence of the precursor peptide ion. The existence of the C-terminal oxazolone structure has been confirmed for b_2^+ and larger species from protonated peptides by our group and others using IRMPD spectroscopy [12–16]. However, more recent experiments [16–22] overwhelmingly indicate that a macro-cyclic *b* ion isomer, or intermediate, can arise through "tail to head" cyclization of linear, oxazolone-terminated *b* ions. The macrocyclic species then can open at various amide bond positions to regain a linear, oxazolone terminated structure, but with the loss of primary sequence information. This type of pathway is referred to as *b*-type scrambling of peptide fragment ions [19], and evidence to support formation of a cyclic b_5 ion from protonated GGGGGR was recently communicated [23].

While apparent "scrambled" b_n ions do not contribute significantly to the product ion distribution(s) generated from protonated precursor peptides, the issue of scrambling will certainly be important in MS^n experiments, such as those reported by Olsen and Mann [24], in which N-terminus containing peptide fragments generated by CID were difficult to rationalize using conventional models of peptide fragmentation. Understanding better the factors that contribute to the tendency for *b* ions to cyclize therefore remains an important goal.

We recently investigated whether apparent sequence scrambling occurs during CID of b_n^+ of differing size. Scrambling was not observed for b_3^+ generated from protonated YAFG and sequence isomers of this peptide. However, scrambling was observed for every larger b_n^+ ion generated from a group of pentapeptides through decapeptide methyl esters. The results of the study clearly established loss of sequence information, consistent with earlier reports of sequence scrambling for b_5^+

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[17–19], and more recently for larger *b* type ions [16, 22]. A later study by Chen and coworkers [15] utilized isotope labeling, infrared photodissociation spectroscopy, gas-phase hydrogen/deuterium exchange (using CH_3OD), and computational methods to determine that *b* ions of varying length using a polyglycine (b_2 to b_8) peptide exist as either oxazolones, macrocyclic species, or a mixture of both. Smaller *b* ions (b_2, b_3) were shown to exclusively demonstrate oxazolone structures, while mid-sized *b* ions (b_4–b_7) were mixed, and the largest *b* ion (b_8 existed primarily as a macrocycle.

Factors contributing to the opening of the macrocycle have also been evaluated by our group. For example, reactions attributable to amino acids with nucleophilic side chains can lead to nonclassic *b* ions. For peptides with amino acids such as Lys (K) and Arg (R), the basic side chain can influence peptide dissociation by providing stabilization through salt bridges [25, 26] or by participating in nucleophilic attacks [26–29]. A more indirect effect would be sequestration of the mobile proton by an amino acid side group, such that suppression of proton migration to populate a reactive configuration inhibits formation of a given product ion. We have shown, however, that model peptides continue to produce sequence informative ions even when obvious mobile protons are immobilized [30]. In this case, mobilization of amide position protons has been invoked to explain product ion (including *b*-type) ion formation [30, 31].

We recently investigated whether selective opening of a putative macrocyclic *b* ion or *b* ion intermediate occurs during CID of b_5^+ for peptides containing amino acid residues with basic, acidic, or amide (polar) side chains [32]. Many notable observations were made during the study of the CID of b_5 ion derived from the YAXFLG, where X = Q, N, D, E, or K, and sequence isomers of the peptides. One observation was that CID of b_5^+ derived from precursor peptides containing polar (amide) side chains (Q, N) caused elimination of NH_3, CO to generate a_5^+, or losses of Q or N residues. Similar results were observed following CID of b_5^+ derived from precursor peptides with acidic side chains (D and E), with losses of water, CO to generate a_5^+, and losses of D or E residues were all observed. CID of b_5^+ from a precursor peptide with lysine (K) at position X led to a dominant loss of H_2O and of the internal K residue. For a series of permuted isomers with Q, nearly identical product ion spectra were generated from b_5^+, regardless of the initial sequence position of the residue, thus establishing a direct influence of opening of the macrocyclic ion by the nucleophilic side group.

Observations made during our study of the possible influence of amino acid side chains on fragmentation patterns of *b*-type ions motivated the experiments reported here. In the present study, fragmentation of b_5^+ generated from a series of model hexapeptides that contain a single arginine (R) residue was investigated. Our primary interest was whether R influences formation of the putative macrocyclic b_5 ion, and in turn the degree to which sequence information is lost in the product ion spectrum. As in our past experiments, two general criteria were used to determine whether scrambling of sequence (through the formation of macrocycle) has occurred: (1) observation of the elimination of internal residues from b_5^+, and (2) generation of identical product ion distributions for b_5^+ derived from peptides with different amino acid sequence. R is the most basic amino acid and, as noted earlier, presence of the residue may influence fragmentation, in part, by sequestering a proton, and thus inhibiting transfer to a cleavage site. In fact, this was one of the key observations made during initial development of the mobile proton model [3, 4]. Investigation of the fragmentation of b_n ions that contain internal R residues (rather than tryptic-type peptides with C-terminal R or L) is also important given the continued use of glutamyl endopeptidases for digestion of proteins for mass mapping [33–36]; these enzymes cleave to the C-terminal side of glutamic or aspartic acid residues depending on experimental conditions [33, 36].

Experimental

Peptide Synthesis and Preparation

All peptides were prepared using conventional solid-phase synthesis techniques [37] utilizing 9-fluorenylmethoxycarbonyl (Fmoc) amino acid loaded Wang resin, Fmoc-protected amino acids, and a custom-built, multiple reaction vessel peptide synthesis apparatus. Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (vol:vol) mixture of HPLC grade MeOH (Aldrich Chemical, St. Louis, MO, USA) and deionized H_2O to produce final concentrations of 10^{-5}–10^{-4} M. Correct sequence was confirmed using CID of metal (Li^+ and Ag^+) cationized versions of the peptides, which is an effective way for determining sequence from the C-terminus [38].

Mass Spectrometry

All ESI mass spectral data were collected utilizing a Finnigan LCQ-Deca ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Peptide solutions were infused into the ESI-MS instrument using the incorporated syringe pump at a flow rate of 5 μL/min. Optimization of the atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octapole voltage offsets, etc.) for maximum $(M + H)^+$ transmission to the ion trap mass analyzer was performed using the auto-tune routine within the LCQ Tune program of the instrument platform. Helium was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

During the initial CID stage (MS/MS or MS^2 stage), the $(M + H)^+$ ions were isolated using an isolation width of 1.2 to 1.8 mass to charge (*m/z*) units. The b_5^+

product ions selected for subsequent CID (MS^3 experiments) were isolated using widths of 1.2>194>1.5 *m/z* units. For each individual MS stage, the width was empirically chosen to produce both the highest precursor ion intensity and ability to isolate a single isotopic peak. The (mass) normalized collision energy (as defined by Thermo) was set between 20% and 25%, which corresponds roughly to 0.55–0.68 V tickle voltage applied to the end cap electrodes with the current instrument calibration. The activation Q, which defines the frequency of the applied rf potential, was set at 0.30 for all CID stages. In all cases, the activation time employed was 30 ms. Spectra displayed represent the accumulation and averaging of at least 30 isolation, dissociation, and ejection/detection steps.

Results and Discussion

In our previous studies, the similarity of product ion distributions for *b* ions derived from precursor peptides and their permuted isomers, and the elimination of internal amino acid residues to generate smaller *b*- and *a*-type ions were used as the principal evidence for *b*-type ion scrambling [15, 17]. As noted earlier, for peptides containing nucleophilic side chains there is strong evidence that the side chains directly influence the opening of the macrocyclic ring and fragment ion patterns generated [18]. During our previous study, it was discovered that only one of the model YAXFLG peptides, namely YARFLG, did not show loss of the internal X residue during CID of b_5^+. All others showed pronounced elimination of the residue at position X, and for X = Q, no dependence of the elimination of the residue on the sequence position was observed. Because of this observed anomaly within the dataset, a series of permuted isomers were synthesized in which the R residue was placed at each of the five possible sequence positions of the b_5^+ ion that would be generated by loss of G from the hexapeptide YARFLG and its permuted isomers. The CID spectra of these b_5 ions were collected and evaluated for possible internal residue losses, as well as similarity of product ion distributions.

Figure 1 shows the CID spectra for b_5^+ derived (by loss of the C-terminal G residue) by CID of the arginine containing permuted isomers with the R residue located at internal sequence positions (LYARFG, YARFLG, and ARFLYG). Examination of each spectrum shows that the product ion distributions generated from the respec-

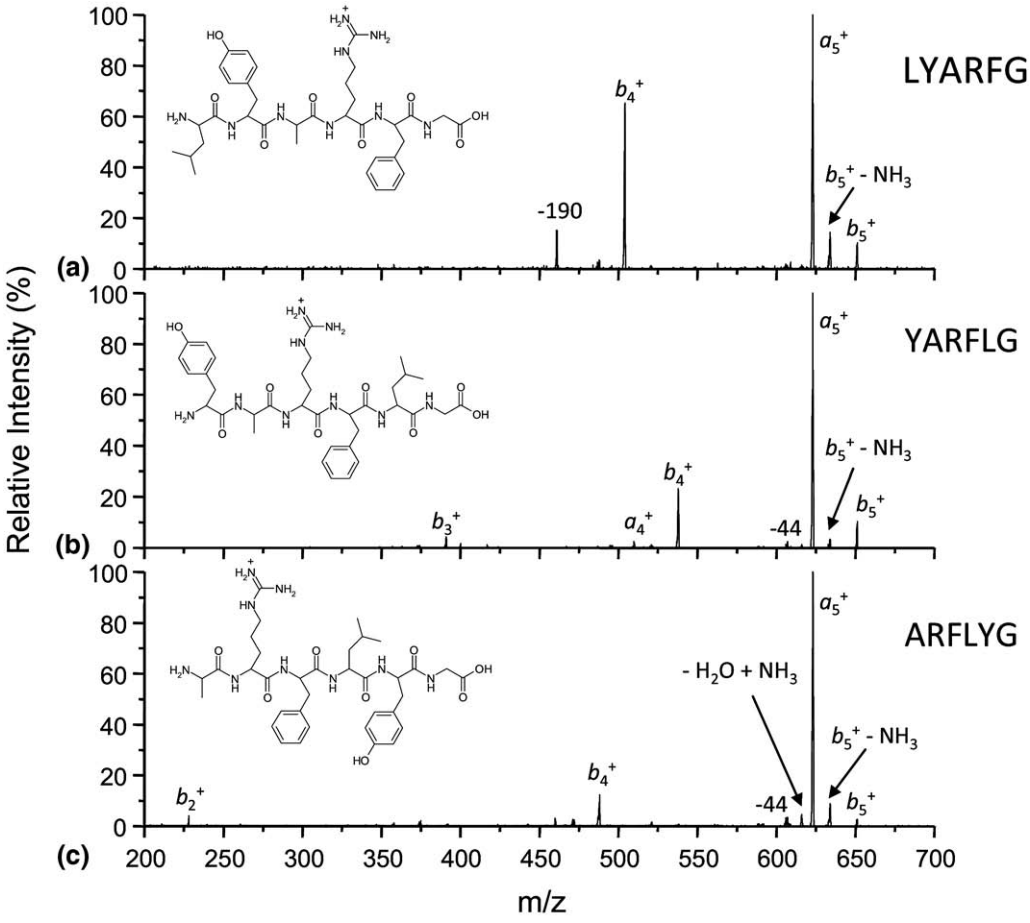


Figure 1. CID of b_5^+ (MS^3) of the YARFLG permuted isomers with internal R residues. The respective $[M + H]^+$ structures are shown.

tive b_5 ions are unique. More importantly, all of the fragment ions generated, regardless of the initial peptide sequence, are either direct sequence ions or ions that are a result of losses of side-chain related fragments: no apparent formation of a cyclic b_5 ion or intermediate, or resulting scrambling of sequence and elimination of internal residues, is observed. We note that species in which the C-terminal most residue is eliminated along with a small molecule such as H_2O might more accurately be described as rearrangement ions rather than direct sequence ions. However, because they do not arise via elimination of internal residues, which would be one of the criteria used to determine whether macrocycle formation and sequence scrambling has occurred, we refer to these species here as direct sequence.

For each b_5 ion, the major fragment ion produced by CID is a_5^+ (through elimination of CO). Other product ions generated by CID of b_5 ion from LYARFG, or $LYARF_{oxa}$ (Figure 1a), include b_5-NH_3, a_5, b_4, and a fragment created by elimination of 190 mass units (u). This last ion is most likely b_4-43, with the additional 43 u attributed to a portion of the R side chain. For CID of b_5^+ derived from YARFLG (Figure 1b), the fragment ions observed include b_5-NH_3, a_5, b_4, a_4, b_3, and a minor ion (less than 5% relative intensity) generated by elimination of 44 u. This ion could likely be attributed to decomposition of the R side chain or an alternate pathway in which the R side chain is participating in the facilitation of the loss as indicated by the location of the amino acid within the peptide sequence, as little is known about the possible alternative pathways for nontryptic peptides with internally positioned R residues. For CID b_5^+ derived from ARFLYG ($ARFLY_{oxa}$, Figure 1c) fragment ions observed include b_5-NH_3, a_5, b_4, b_2, b_5-(H_2O + NH_3), and like YARFLG, a product generated by elimination of 44 u.

Regardless of which permuted sequence isomer was investigated (YARFLG, LYARFG, or ARFLYG), none of the product ions generated following CID of b_5^+ are attributable to reactions in which an internal residue was observed. This observation is in stark contrast to those reported in our earlier studies [19, 21], and those of Harrison [22], in which several fragment ions generated by decomposition of b_5^+ ions derived from peptides such as YAGFLG and larger peptides could only be formed through the elimination of residues from internal sequence positions. It appears, therefore, that the R

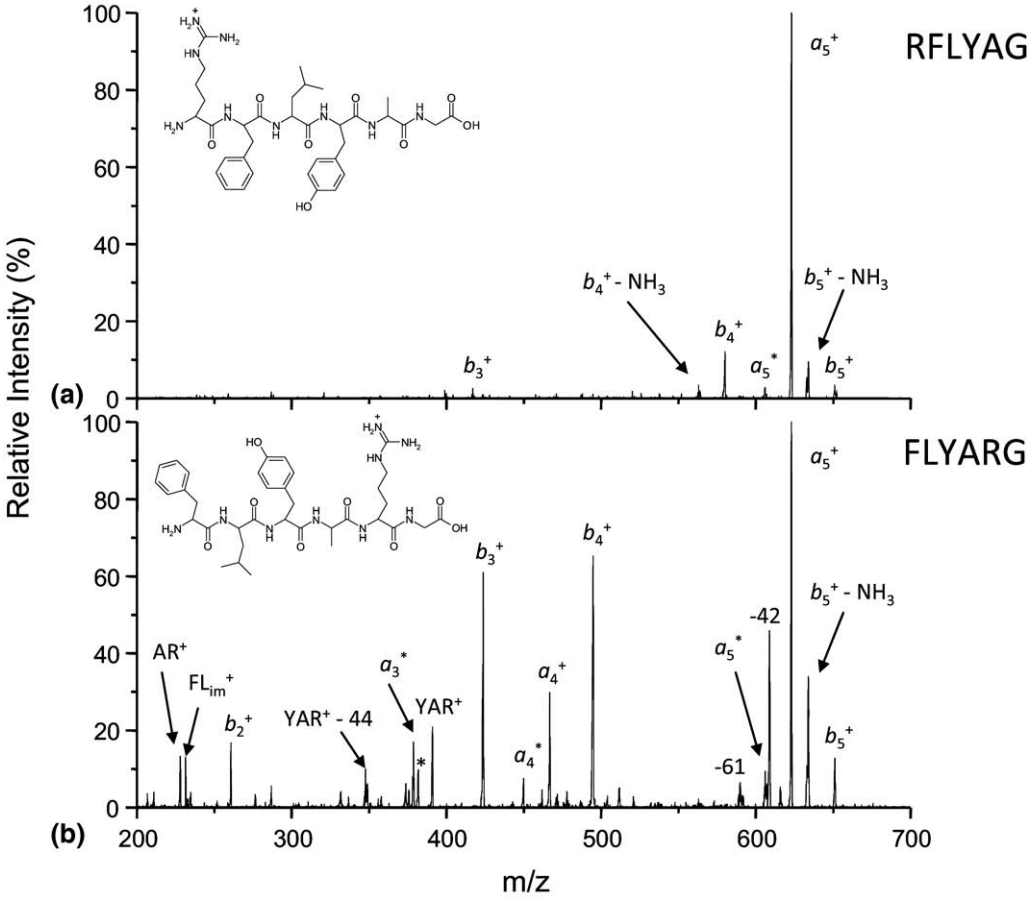


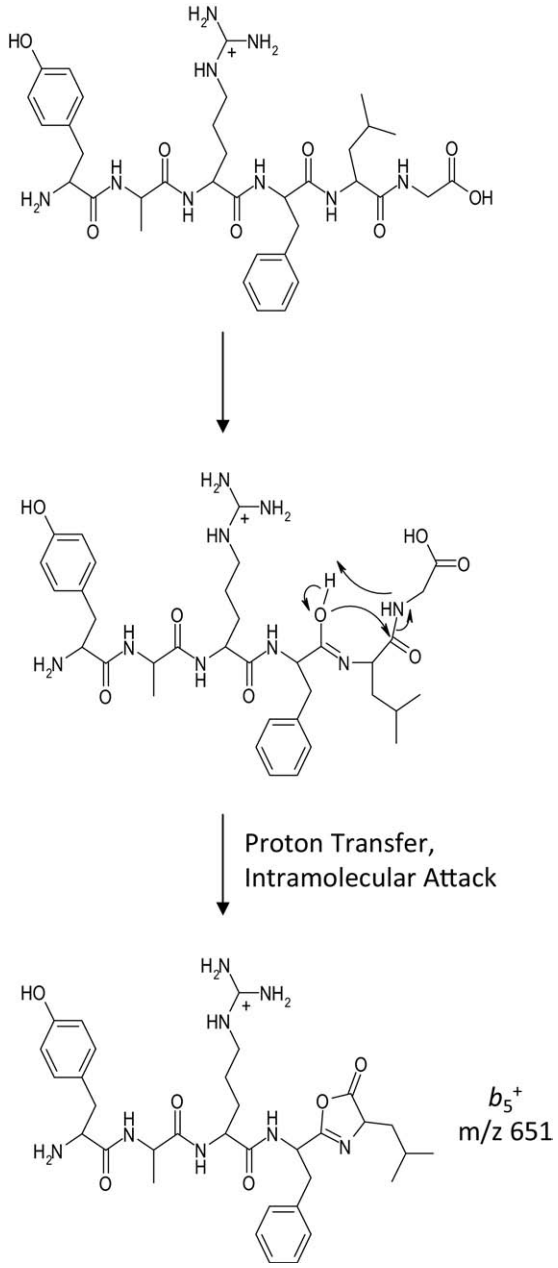
Figure 2. CID of b_5^+ (MS^3) of the YARFLG permuted isomers with external R residues. The respective $[M + H]^+$ structures are shown.

side chain inhibits formation of macrocyclic b_5^+ and that CID of this ion instead generates direct sequence ions that allow for characterization of the respective peptides. Further experiments (data not shown) also show that sequencing via multiple-stage (MS^n) CID was possible through monitoring the serial production of b_n^+ and a_n^+ ions, and suggest that late stage macrocycle formation is also inhibited.

Figure 2 shows the b_5^+ CID spectra for the two remaining permuted isomers in which the R residues are positions at the "external" N-terminal and C-terminal positions, i.e., RFLYAG (Figure 2a) and FLYARG (Figure 2b), respectively. Inspection of the spectra in Figure 2 shows that the product ion distributions are again unique. As for the permuted peptide isomers with R positions at internal R sequence positions, the major fragment ion generated from b_5^+ derived from either RFLYAG or FLYARG is a_5^+. Other fragment ions generated by CID b_5 ion derived from RFLYAG ($RFLYA_{oxa}$, Figure 2a) are all direct sequence ions and include b_5-NH_3, a_5, a_5^*, b_4, b_4-NH_3, and b_3.

More complex, however, is the CID spectrum of the b_5^+ derived from FLYARG (Figure 2b). Fragment ions for this peptide include b_5-NH_3, a_5, a_5^*, b_4, a_4, a_4^*, b_3, a_3^*, b_2, YAR, YAR-44, AR, FL_{im}, as well as several other ions as a result of a loss of 42 and 61 u. The loss of both 42 and 61 u is likely attributable to decomposition of the R side chain. The reason for the added complexity of fragmentation for b_5^+ from protonated FLYARG is not known at this time, but might be attributable to the possible the existence of multiple b_5 structural isomers. For example, one might feature an extended structure with C-terminal oxazolone group, while the other an extended structure with larger macrocyclic C-terminal group formed by attack by the arginine side chain.

Clearly, detailed mechanistic studies, as well as application of density functional theory calculations to identify key reactive configurations and transition states, would aid the assessment of how the fragmentation patterns for b_5^+ derived from FLYARG, and from each b_5 ion, are affected by peptide sequence. A comprehensive DFT study is outside our area of expertise and beyond the scope of the current communication. The dominant fragment ion for all permuted isomers is the a_5^+, but also observed are direct sequence ions, including b, a, and ions related to arginine side-chain peptide fragmentation pathways. It is not clear whether the influence of the R residue is on the possible rearrangement of an initial oxazolone-type ion, after its formation, to furnish the macrocycle or, instead, on direct formation of a cyclic b_5 species directly from $(M + H)^+$. For the former case, it is conceivable that the R side group stabilizes through intramolecular H-bonding a structure that prevents rearrangement and the "tail to head" cyclization step. For the latter case, sequestering of the "mobile" proton by the R side group would require mobilization and transfer of an amide position proton. Such a process has been invoked to explain formation of b_2^+ from a model glycyl-glycine methyl ester peptide that featured and N-terminal pyridine group introduced to sequester the mobile proton, with evidence supplied by CID with isotope labeling and IRMPD spectroscopy [30]. More recently, Paizs and coworkers invoked the mobilization of an amide position H atom through formation of an iminol tautomer to explain the fragmentation of peptides with R residues [31]. Using the model proposed by Paizs and coworkers, we can suggest that formation of the b_5^+ as an oxazolone product from YARFLG may proceed as outlined in Scheme 1. Mobilization of the amide proton from the L residue, through an iminol tautomer, would



Scheme 1. Pathway showing the proton sequestration of the arginine side chain of YARFLG and resulting amide mobilization reaction, leading to the b_5 ion.

ultimately yield the b_5 ion via nucleophilic attack and formation of a deprotonated oxazolone ring.

Conclusions

To summarize, the objective of this study was to determine whether a macrocyclic *b* ion or *b* ion intermediate occurs during CID of b_5^+ for peptides containing an arginine residue. It is clear from comparison of the CID spectra generated from each of the b_5^+ species derived from the permuted sequence isomers of YARFLG that unique product ion distributions are generated, without pronounced elimination of internal residues, thus suggesting that the presence of R inhibits formation of macrocyclic b_5^+ from this small group of model peptides. The exact role of the R residue in the inhibition of macrocycle formation and/or sequence scrambling is not known. Possibly, the high basic guanidine side group sequesters a proton necessary for facile formation of the macrocycle. The general influence of R on the formation of macrocyclic *b* ions that are larger than those examined in this study is also not known, and is currently under investigation using a larger set of peptides. In addition, it will be interesting to study the effect of R in close proximity to residues with acidic side groups (D, E) and the possible role of salt-bridging structures on the fragmentation reactions of *b*-type ions.

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